

GluR2 ligand-binding core complexes: importance of the isoxazolol moiety and 5-substituent for the binding mode of AMPA-type agonists

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Abstract X-ray structures of the GluR2 ligand-binding core in complex with (*S*)-Des-Me-AMPA and in the presence and absence of zinc ions have been determined. (*S*)-Des-Me-AMPA, which is devoid of a substituent in the 5-position of the isoxazolol ring, only has limited interactions with the partly hydrophobic pocket of the ligand-binding site, and adopts an AMPA-like binding mode. The structures, in comparison with other agonist complex structures, disclose the relative importance of the isoxazolol ring and of the substituent in the 5-position for the mode of binding. A relationship appears to exist between the extent of interaction of the ligand with the hydrophobic pocket and the affinity of the ligand.

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Key words: AMPA analogue; X-ray crystallography; Ionotropic glutamate receptor; Ligand-binding core; Agonist

1. Introduction

Glutamate mediates synaptic transmission between nerve cells via binding to glutamate receptors (GluRs), and is responsible for most fast excitatory signaling within the mammalian central nervous system (CNS). The ionotropic GluRs (iGluRs) form ligand-gated ion channels, and are divided into three heterogeneous classes: 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), kainic acid, and *N*-methyl-D-aspartic acid (NMDA) receptors [1]. The functional iGluR is a tetramer composed of dimers-of-dimers [2] and references therein). The iGluRs are involved in various aspects of normal brain function, and are important for the synaptic plasticity implicated in learning and memory formation [3].

The two segments S1 and S2 have been shown to constitute the ligand-binding core, to which the neurotransmitter is bound (Fig. 1A). Previous studies have demonstrated that

the S1 and S2 segments are necessary and sufficient to obtain a pharmacological profile comparable to that of the full-length membrane-bound receptor [4,5].

A recombinant, soluble form of the ligand-binding core of the AMPA-receptor subunit GluR2 (GluR2-S1S2J) has been produced by omitting the N- and C-terminal domains and by substituting all transmembrane regions with a short hydrophilic linker between the S1 and S2 segments [6,7]. Crystal structures of the ligand-binding core of the apo form, of one antagonist and several agonist complexes have been published [6–8].

The ligands are bound in a cleft between two domains (D1 and D2), where D1 primarily is composed of segment S1, and D2 primarily of segment S2 [6]. Comparison of the apo structure with the ligand-bound complexes has disclosed that binding of different ligands induces variable degrees of domain closure. Interestingly, (*S*)-glutamate and (*S*)-AMPA adopt different binding modes [7]. However, AMPA analogues with large 5-substituents at the isoxazole moiety as (*S*)-2-amino-3-[3-hydroxy-5-(2-methyl-2*H*-tetrazol-5-yl)-4-isoxazolyl]propionic acid ((*S*)-2-Me-Tet-AMPA) has been shown to adopt a binding mode similar to that of glutamate [8].

In this study, the importance of the isoxazolol moiety, and in particular the presence of a substituent at the 5-position, has been investigated in relation to the mode of ligand binding. The AMPA analogue 2-amino-3-(3-hydroxy-4-isoxazolyl)propionic acid (Des-Me-AMPA, Fig. 1B), which has a hydrogen atom in the 5-position, shows relatively high AMPA receptor affinity ($IC_{50} = 0.27 \mu M$ for the racemate [9]). Two high-resolution X-ray structures of GluR2-S1S2J in complex with (*S*)-Des-Me-AMPA in the presence and absence of zinc ions are presented and are compared with other agonist structures.

2. Materials and methods

2.1. Materials

(*S*)-Des-Me-AMPA was a kind gift from L. Brehm and B. Nielsen (the asymmetric synthesis of the compound remains to be published). The recombinant GluR2-S1S2J construct was expressed, refolded, and purified essentially as reported [10].

2.2. Binding experiments

Ligand binding was performed as described previously [5]. Briefly, for saturation binding refolded GluR2-S1S2J protein (0.08 mg/ml) was incubated for 1 h on ice with 1–200 nM [³H]AMPA (11.1 Ci/mmol) in binding buffer (100 mM thiocyanate, 2.5 mM CaCl₂, and 30 mM Tris-HCl, pH 7.2) to a total volume of 500 μ l. Competition

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Abbreviations: GluR, glutamate receptor; iGluR, ionotropic glutamate receptor; AMPA, 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid; NMDA, *N*-methyl-D-aspartic acid; Des-Me-AMPA, 2-amino-3-(3-hydroxy-4-isoxazolyl)propionic acid; 2-Me-Tet-AMPA, 2-amino-3-[3-hydroxy-5-(2-methyl-2*H*-tetrazol-5-yl)-4-isoxazolyl]propionic acid

experiments were performed under similar conditions, using 20 nM [^3H]AMPA and 0.02 nM–50 μM (*S*)-Des-Me-AMPA. Non-specific binding was determined in the presence of 1 mM glutamate. The binding experiments were performed in triplicate.

2.3. Co-crystallization experiments

Crystallization was carried out at 6°C using the hanging-drop vapor diffusion method. Protein and ligand were mixed at 6°C 3–4 h prior to crystallization experiments. The final mixture contained 8 mg/ml GluR2-S1S2J and 3 mM (*S*)-Des-Me-AMPA in 10 mM HEPES, pH 7.4, 20 mM NaCl, 1 mM EDTA, giving a molar ratio of 1:11. Drops comprising 1 μl protein–ligand solution and 1 μl reservoir solution were employed. Crystals were obtained both in the presence and absence of zinc ions. The zinc-containing crystals were obtained from a reservoir solution of 0.05 M $\text{Zn}(\text{OAc})_2$, 17% polyethylene glycol (PEG) 8000, 0.1 M cacodylate, pH 6.5, whereas the zinc-free crystals were obtained from a reservoir solution of 0.1 M Li_2SO_4 , 20% PEG8000, 0.1 M cacodylate, pH 5.2.

2.4. Data collection and processing

Crystals of GluR2-S1S2J in complex with (*S*)-Des-Me-AMPA were flash-cooled to 100 K using 18–20% glycerol added to the reservoir solutions, as a cryoprotectant. Complete synchrotron data for the zinc-free and for the zinc-containing crystals were collected at the EMBL beamlines X11 and BW7B, DESY, Hamburg, equipped with a MAR CCD and a MAR IP detector, respectively. Data processing was performed using the HKL programs DENZO and SCALEPACK [11], and the CCP4 suite of programs [12]. Statistics are listed in Table 1.

2.5. Structure determination and refinement

Both structures were solved using the molecular replacement (MR) approach, employing the program AMoRe [13] from CCP4. The zinc-free complex was solved using the complex of GluR2-S1S2J and (*S*)-2-Me-Tet-AMPA (protein atoms only) [8] as a search model. A clear solution comprising three molecules was obtained. Subsequently, automated model building was performed with the program ARP/wARP [14]. This resulted in the tracing of 94% of the residues, including a fourth molecule which was not obtained with MR. The missing residues were inserted by manual model building in the program O [15]. All three molecules in the zinc-containing structure were localized with AMoRe using the zinc-free form as a search model. ARP/wARP built 89% of the residues, and the remaining residues were built manually. The structures were subjected to rebuilding in O and refinements in CNS [16]. Ligand molecules, water molecules, zinc ions, sulfate ions, and glycerol were gradually introduced into the structures. Statistics are listed in Table 1.

The Fo–Fc electron densities for the ligand molecules were in all cases very well defined and the ligands were unambiguously modelled into it. Initially, the ligand was geometry optimized with molecular mechanics using the MMFFs forcefield in MacroModel 7.2, including GB-SA aqueous solvation [17]. Topology and parameter files for the ligand, used in CNS and in O, were generated by the HIC-Up server [18].

The HINGEFIND script [19], which is part of the programVMD [20], was employed for analysis of domain closure. Figures were prepared using the programs Molscript [21] and Raster3d [22].

2.6. Protein data bank accession numbers

The atomic coordinates and structure factor amplitudes of the GluR2-S1S2J (*S*)-Des-Me-AMPA complexes have been deposited with the RCSB Protein Data Bank (ID codes 1MQD and 1MS7).

3. Results and discussion

3.1. Characterization of GluR2-S1S2J

Saturation binding of [^3H]AMPA and competitive displacement of [^3H]AMPA by (*S*)-Des-Me-AMPA was performed in order to compare the pharmacology of the GluR2-S1S2J construct with the pharmacology of the full-length receptor (Fig. 2). The pharmacological profile of GluR2-S1S2J with an IC_{50} of 0.17 μM for (*S*)-Des-Me-AMPA is comparable to that of the wildtype full-length GluR2 receptor (0.09 μM [Stensbøl et al., to be published]).

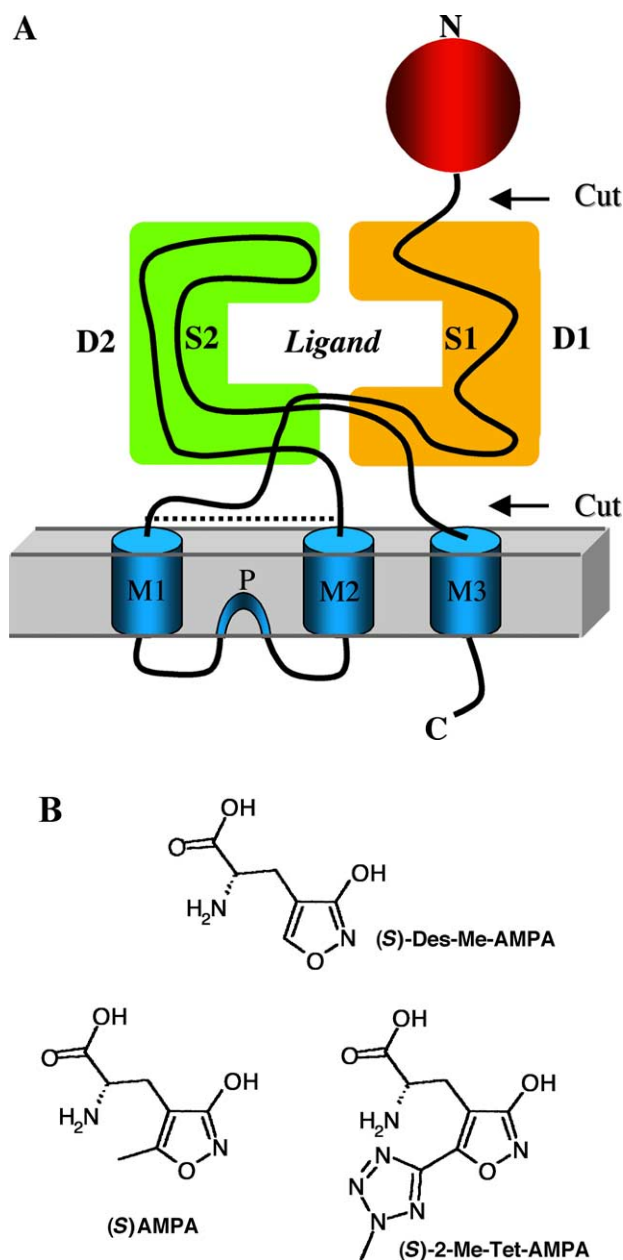


Fig. 1. A: The GluR2-S1S2J construct, comprising the ligand-binding core of the AMPA-receptor subunit GluR2, is composed of segments S1 and S2, joined by a linker (dashed line). The two domains D1 and D2 are shown in yellow and green, respectively. The amino-terminal domain is outlined as a red sphere, and integral membrane parts are blue. B: The chemical structures of (*S*)-Des-Me-AMPA, (*S*)-AMPA and (*S*)-2-Me-Tet-AMPA.

3.2. Interactions of (*S*)-Des-Me-AMPA with GluR2-S1S2J

The overall structure of the ligand-binding core of GluR2 has been described previously [6]. In this study, seven complex molecules (MolA–C of the zinc-containing structure and MolD–G of the zinc-free structure) have been built and refined. All molecules form dimers in the crystal. MolA is used as reference molecule throughout the text. The seven molecules are very similar when superimposed on MolA (r.m.s. difference 0.27–0.37 Å on all C α atoms for MolC–G and 0.50 Å for MolB; for the latter, the largest differences were observed in loop 1 [6]). The other agonist complexes with (*S*)-glutamate (MolA), (*S*)-AMPA (MolA) [7] and (*S*)-2-Me-Tet-

Table 1
Crystal data, data collection, and refinement statistics

	Crystals without zinc	Crystals with zinc
Space group	P2	P2 ₁ 2 ₁ 2
Unit cell parameters (Å, °)	$a = 105.4$, $b = 47.5$, $c = 123.9$, $\beta = 113.7$	$a = 113.7$, $b = 162.9$, $c = 47.1$
Molecules/a.u.	4	3
Resolution range (Å) ^a	20–1.46 [1.49–1.46]	20–1.97 [2.02–1.97]
Unique reflections	191 888	62 567
Average redundancy	3.9	5.2
Completeness (%)	99.0 [95.5]	99.5 [94.6]
R_{merge} (%)	4.2 [38.4]	11.8 [65.2]
$\ \sigma(I) \ $	26.7 [2.4]	12.1 [2.2]
Non-hydrogen atoms	10 796	7 287
Amino acid residues	1 032	774
Ligand/Zn ²⁺ /glycerol/sulfate	4/0/1/3	3/6/0/0
Water molecules	2 651	1 187
R_{work} (%)	18.0	19.1
R_{free} , 2% (%)	19.2	21.2
r.m.s. bond lengths (Å)/angles (°)	0.005/1.3	0.007/1.5
Residues in allowed regions of Ramachandran plot (%) ^b	99.8	99.6
Average B -values (Å ²) for protein/ligand atoms	15.9/11.5	21.3/20.1

^aThe values in brackets correspond to the outermost resolution shell.

^bThe Ramachandran plot was calculated according to Kleywegt and Jones [26].

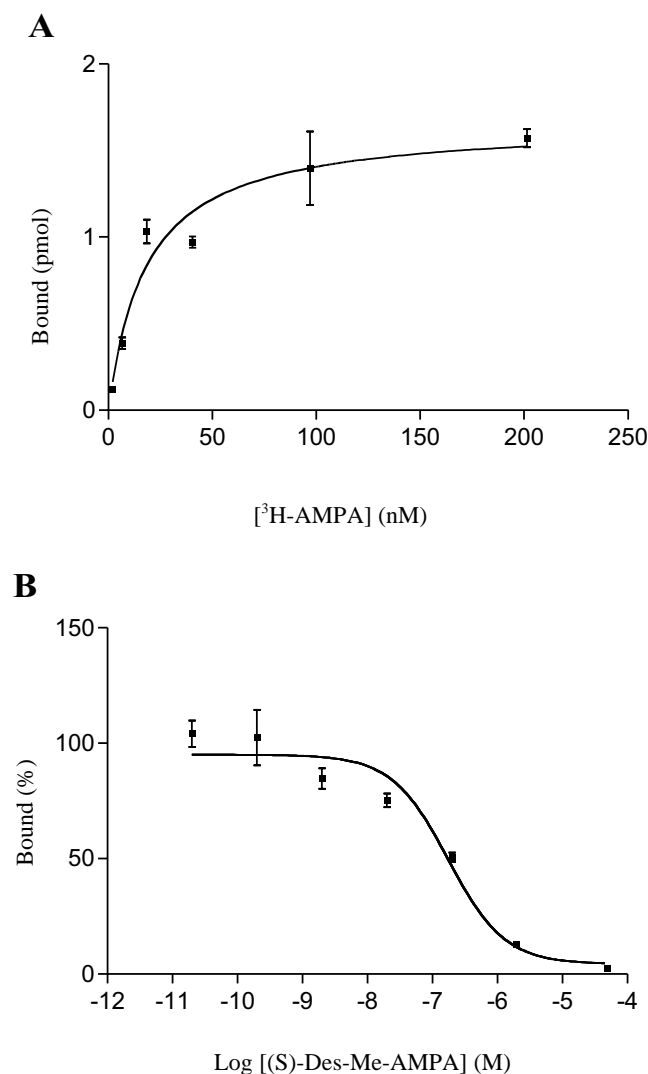


Fig. 2. Binding characteristics of the GluR2-S1S2J protein. A: Saturation binding curve for [³H]AMPA. The calculated K_d is 18.1 nM (± 4.6 nM) and B_{max} is 1.66 pmol (± 0.1). B: Displacement of [³H]AMPA by (*S*)-Des-Me-AMPA. IC_{50} is 0.17 μ M (0.086–0.354 μ M).

AMPA (MolA) [8] used for comparison in this study superimpose with r.m.s. differences of 0.35–0.42 Å.

(*S*)-Des-Me-AMPA is observed to bind to the ligand-binding site in a very similar manner in all molecules. The interactions between ligand and the ligand-binding core include hydrogen bonds, ionic interactions and van der Waals interactions (Fig. 3A, Table 2). The α -carboxylate group interacts with Thr480 and Arg485 of D1, and with Ser654 of D2. Furthermore, the α -carboxylate is at a hydrogen-bonding distance to the water molecule W4 (water molecules are numbered according to [8]). The α -ammonium group is positioned in a tetrahedral network of interactions to Pro478 and Thr480 of D1, and Glu705 in D2. This overall arrangement of the α -amino acid part of the ligand is in accordance with the previously reported agonist complex structures [6–8]. The 3-hydroxy group interacts with Thr655 from D2 and with W2 and W4, which mediate indirect interactions to Leu650, Ser654, Thr655 and Leu703. The isoxazole ring nitrogen is at a hydrogen-bonding distance to Glu705 and to W3, which is further connected to the side chains of Thr686 and Tyr702 in D2.

3.3. The importance of the isoxazolol moiety and of the 5-substituent for ligand-binding mode

Until now, two modes of binding of an agonist to GluR2-S1S2J have been observed, the so-called AMPA and glutamate-binding modes [7,8]. The interactions of the 3-hydroxy group in (*S*)-Des-Me-AMPA are similar to those observed in the structure of the (*S*)-AMPA complex. Of special interest is the presence of the water molecule W4, which has so far only been observed in the AMPA structure, and which tethers the agonist to the protein residues Ser654 and Thr655 (Fig. 3B). In contrast, (*S*)-glutamate and (*S*)-2-Me-Tet-AMPA are located at positions closer to D1 in the ligand-binding cleft. A γ -carboxylate oxygen and a hydroxy oxygen, respectively, here mimic the water molecule W4 observed in the (*S*)-AMPA structure, and are able to make direct interactions to the same protein residues.

As (*S*)-Des-Me-AMPA adopts the AMPA mode of binding to the receptor, it may be concluded that it is the isoxazolol

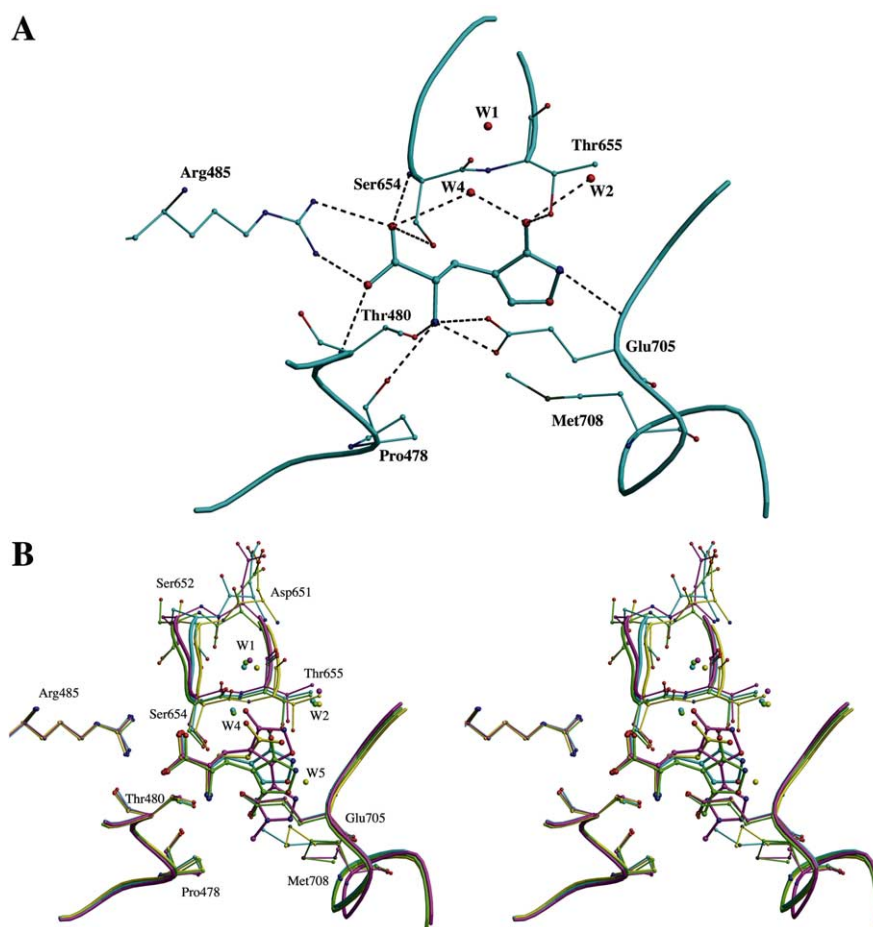


Fig. 3. The ligand-binding site of GluR2-S1S2J. A: Interactions between GluR2-S1S2J and (*S*)-Des-Me-AMPA. Dashed lines indicate potential hydrogen bonds/ionic interactions (up to 3.3 Å). Water molecules are displayed as red spheres. Nitrogen atoms are shown in blue, oxygen atoms in red, and sulfur atoms in dark green. B: Superimposition of the (*S*)-Des-Me-AMPA (cyan), (*S*)-AMPA (green), (*S*)-2-Me-Tet-AMPA (magenta) and (*S*)-glutamate (yellow) complexes (shown in stereo). For all structures MolA is used as a representative. The conformation of Met708 in the glutamate complex is unique for MolA, as the other two molecules have extended Met708 chains.

moiety, and not the 5-methyl group, which directs the binding mode. However, AMPA analogues with larger 5-substituents as (*S*)-2-Me-Tet-AMPA seem to favor the glutamate-binding mode [8].

3.4. Met708 exerts an induced fit depending on the structure of the bound agonist

By studying Fig. 3B, it is evident that the side chain of Met708 from D2 adopts different conformations depending on which ligand is bound. When a hydrogen atom is present in the 5-position, as in (*S*)-Des-Me-AMPA, Met708 has an extended side-chain conformation, to maximize the stabilization of the ligand. Also in the (*S*)-AMPA structure, the conformation of Met708 is optimized for favorable van der Waals interactions with the isoxazolol moiety and the 5-methyl substituent. In contrast, only weak stabilization by Met708 is possible when glutamate is bound, as this agonist is located in the upper part of the binding site. In the (*S*)-2-Me-Tet-AMPA structure, Met708 adopts a conformation in which the side chain partly points away from the 5-tetrazole ring, thereby optimizing interactions with this ring. In this complex, the backbone conformation around Met708 is also slightly different from the other structures, pulling Met708 outwards. This observation leads us to conclude that Met708 exerts an

induced fit, in order to maximize the van der Waals stabilization of the bound ligand.

3.5. Relationship between the interaction with the partly hydrophobic pocket and the affinity of the ligand

A partly hydrophobic pocket exists in the ligand-binding cleft of GluR2-S1S2J, which includes the above mentioned Met708 [8]. The isoxazolol ring of (*S*)-Des-Me-AMPA is involved in van der Waals interactions with Tyr450 and Met708, while the presence of a methyl group in the 5-position of the isoxazole ring of (*S*)-AMPA makes an additional van der Waals interaction to Pro478 possible. (*S*)-2-Me-Tet-AMPA, which has a large tetrazole ring in the 5-position, is deeply imbedded in the pocket and is also in van der Waals contact with Glu402 and Tyr405. However, the pocket is not large enough to completely accommodate the tetrazole ring, and this forces the ligand further up in the binding pocket to adopt the glutamate-binding mode. Glutamate is not able to interact with the hydrophobic pocket. Interestingly, there seems to be a relationship between the extent of interaction between the ligands and the hydrophobic pocket and the affinity (IC_{50}) of the ligands: (*S*)-glutamate $0.50 \mu M$ [23] < (*S*)-Des-Me-AMPA ($0.09 \mu M$) < (*S*)-AMPA ($0.02 \mu M$) [23] < (*S*)-2-Me-Tet-AMPA ($0.009 \mu M$) [8].

Table 2
Hydrogen bonds and ionic interactions between (S)-Des-Me-AMPA and GluR2-S1S2J

	(S)-Des-Me-AMPA			(S)-Des-Me-AMPA				(S)-AMPA	(S)-2-Me-Tet-AMPA	(S)-glutamate
	Zn ²⁺ present			No Zn ²⁺ present						
	MolA	MolB	MolC	MolD	MolE	MolF	MolG	MolA	MolA	MolA
Carboxylate oxygen 1										
Thr480 N	3.1	3.0	3.0	3.0	3.1	3.1	3.1	3.0	2.9	3.0
Arg485 NH1	2.9	2.9	2.8	2.7	2.7	2.7	2.7	2.8	2.8	2.8
Carboxylate oxygen 2										
Arg485 NH2	3.0	3.0	3.1	2.9	3.0	2.9	2.9	3.0	2.8	2.9
Ser654 N	2.9	3.0	2.8	2.9	2.9	3.0	2.9	3.0	2.9	2.9
Ser654 OG	3.3	3.4	3.2	3.3	3.4	3.4	3.3	3.3	3.3	3.3
W4	3.1	3.0	3.0	3.1	3.2	3.1	3.1	2.9	–	–
Ammonium group										
Pro478 O	2.9	2.9	2.6	2.9	2.9	2.9	2.9	2.7	3.0	2.7
Thr480 OG1	2.8	2.9	2.9	2.9	2.9	2.9	2.9	3.0	3.0	3.0
Glu705 OE1	2.5	2.5	2.6	2.7	2.7	2.7	2.7	2.7	2.7	2.7
Glu705 OE2	3.0	3.4	3.1	3.1	3.2	3.1	3.1	3.3	3.5	3.2
3-Hydroxy oxygen										
Ser654 N	–	–	–	–	–	–	–	–	3.2	3.3 ^a
Thr655 OG1	2.5	2.6	2.6	2.6	2.6	2.6	2.7	2.7	3.5	2.7 ^b
Thr655 N	–	–	–	–	–	–	–	–	3.1	3.2 ^a
W1	–	–	–	–	–	–	–	–	2.8	3.1 ^a
W2	2.9	2.8	3.1	3.0	2.9	2.9	2.9	3.0	–	3.0 ^b
W4	2.3	2.4	2.3	2.5	2.6	2.6	2.6	2.5	–	–
W5	–	–	–	–	–	–	–	–	–	2.8 ^b
Isoxazole ring nitrogen										
Thr655 OG1	–	–	–	–	–	–	–	–	2.8	–
Glu705 N	3.1	3.1	3.2	3.3	3.2	3.2	3.2	3.1	–	–
W2	3.5	–	–	–	–	–	–	–	2.9	–
W3	3.0	2.9	3.0	3.1	3.1	3.1	3.1	3.0	–	–

^aContacts to γ -carboxylate oxygen 1.

^bContacts to γ -carboxylate oxygen 2.

3.6. The peptide flip

Multiple conformations have been observed for the Asp651–Ser652 peptide bond. The peptide bond is flipped in the structures of (S)-AMPA and (S)-2-Me-Tet-AMPA, compared to the conformation observed in the apo, kainate, and DNQX structures [6–8]. In the (S)-glutamate structure, this peptide bond apparently exists in both conformations. A correlation between peptide flip and relative domain closure has been suggested, as a large degree of closure (e.g. 21° for (S)-AMPA relative to the apo structure) has been observed in conjunction with a flipped peptide bond, in contrast to less closed structures (e.g. 12° in kainate).

However, in none of the seven molecules in the asymmetric units of the two complexes with (S)-Des-Me-AMPA do the electron densities indicate that the peptide bond between Asp651 and Ser652 is flipped, even though this ligand induces almost the same domain closure as (S)-AMPA (19–21° for the seven molecules). Thus, no clearcut correlation appears to be present, and the importance of this feature remains to be explored. Neither of the residues Asp651 and Ser652 are in direct contact with the ligands, but the surrounding residues Leu650 and Ser654 make hydrogen bonds/van der Waals interactions with the agonists.

3.7. The effect of zinc and sulfate ions on the complex

The structure of GluR2-S1S2J was determined both in the presence and in the absence of zinc ions, as it has previously been observed that micromolar concentrations of zinc ions can potentiate currents from native and recombinant receptors [24,25].

Six zinc ions were identified in the zinc-containing complex,

coordinating all six His residues in the asymmetric unit. Five zinc ions are located at interprotomer contact sites, while the last one is coordinated by intraprotomer contacts. The zinc ions only seem to induce small local changes in side-chain and backbone conformations in this ligand complex, and the degree of domain closure observed in the two structures is very similar.

The ions coordinate to the following residues: Zn1 to HisA412, HisB412, and GluB419; Zn2 to GluA431 and HisA435; Zn3 to AspA454 and HisC412; Zn4 to GluB431, HisB435 and GluC678; Zn5 to GluB678, GluC431 and HisC435. Zn6 is located next to Zn5 and only coordinates to water molecules. These interactions are similar to those observed previously [7,8]. Three sulfate ions were located in the zinc-free structure. SO₄1 coordinates to ArgB660, SO₄2 to ArgA675, and SO₄3 to ArgA684, ArgD660, TrpD671 and ArgD675. Also, these ions seem to induce only small, local effects.

4. Conclusions

This study leads us to conclude that: (1) The size of the substituent in the 5-position of the isoxazolol ring is a major determinant of the ligand-binding mode for AMPA-type agonists. A large substituent results in a glutamate-like binding mode, whereas small or no substituents leads to AMPA-like binding. The isoxazolol moiety is not solely able to control the binding mode. (2) A relationship appears to be present between the extent of interaction of the 5-substituent with a partly hydrophobic pocket and the affinity of the ligands. (3) (S)-Des-Me-AMPA stabilizes a fully closed (21°) confor-

mation without introduction of the Asp651–Ser652 peptide flip.

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